"Structural and Molecular Studies on Plant Serine Protease Inhibitors"

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Title of the Thesis: Structural and Molecular Studies on Plant Serine Protease Inhibitors

Structural and Molecular Studies on Plant Serine Protease Inhibitors

Proteases are ubiquitous in nature, catalysis the hydrolysis of α-peptide bond in proteins. The proteolytic enzymes or proteases are classified into seven distinct classes; a) serine proteases b) metallo proteases c) aspartic proteases d) cysteine proteases e) threonine f) glutamic and g) asparagine peptide lyases. The classification is based on the interaction of protease catalytic residue with specific inhibitors. A number of studies have elucidated the role of proteases in human diseases such as cancer, thrombotic and inflammatory disorders (Cheronis and Repine, 1993; Horl and Heidland, 1987). Given that they are involved in the pathogenesis of various diseases, they could be good therapeutic targets with their specific protease inhibitors (PI).

Serine proteases

Serine proteases are involved in enormous number of physiological processes such as blood clotting, immune responses, cell death and tissue healing (Losso, 2008. Serine proteases are extensively involved in immunological responses and pro-inflammatory actions. Inflammation is an important contributor to the pathogenesis of tissue injury and disease. Polymorphonuclear cells (PMNs) are the first inflammatory cells to enter injured tissue during inflammation (Kobayashi *et al*., 2005). The serine proteases cathepsin G, human leucocyte elastase and proteinase 3 are major contents of neutrophils and are released at sites of inflammation. Recent studies provided evidence that these proteases activate pro-inflammatory cytokines leading to the activation of different receptors. Neutrophil serine proteases might therefore be important regulators of inflammatory processes and are interesting targets for new therapeutic approaches against inflammatory disorders (Wiedow and Meyer-Hoffert 2005).

The importance of serine proteinases in the development of airway diseases has been emphasized in recent years. Of particular importance is the potential role of tryptase,

thrombin and elastase in development of asthma, in which these serine proteinases were not only been over-secreted, but also found to play a role in induction of cytokine hypersecretion in airways.

Exposure to the agricultural/organic dust is a risk factor for the development of respiratory symptoms and chronic lung diseases. Lung epithelium plays important roles in the control of immune and inflammatory responses to environmental agents to maintain lung health (Boggaram *et al.*, 2016). Cellular and molecular mechanisms mediating lung inflammatory responses to agricultural dust are not yet fully understood (Gottipati *et al*., 2014). The potential mechanism, through which these serine proteinases carry out their actions in respiratory tract, remains unclear.

Serine protease inhibitors

Serine protease inhibitors are classified into different categories based on their substrate specificity, particularly by the type of residue found in PI as either trypsin- like (positively charged amino acid residue), elastase- like (small hydrophobic amino acid residue) or chymotrypsin-like (large hydrophobic residue) (Cheronis and Repine, 1993). Serine protease inhibitors play an effective role in treatment of inflammatory diseases by modifying various inflammatory pathways. A serine protease inhibitor 4-(2-aminoethyl)-benzene sulfonyl fluoride (AEBSF), suppresses inflammatory cytokines and related transcription factors in splenocytes from estrogen-treated mice (Karpuzoglu *et al*., 2011). Alpha-1-antitrypsin (AAT) is a serine protease inhibitor in plasma. Overexpression of AAT in transgenic mice with experimental autoimmune encephalomyelitis (EAE) suppresses clinical disease and inflammation in the CNS. In one report, production of pro-inflammatory cytokines such as IL-17, IL-1β and IL-6 decreased, and the number of Foxp3 Treg in spleens increased compared to wild type control mice (Subramanian *et al.*, 2011).

Neutrophil and mast cell serine proteases play a role in inflammatory pulmonary diseases such as asthma. A synthetic dual inhibitor of cathepsin G and mast cell chymases called RWJ-355871 has demonstrated a potent suppressive effect in allergic inflammatory diseases of the respiratory system (Maryanoff *et al*., 2010). The use of AEBSF has demonstrated an inhibitory effect in inflammation and attenuated disease severity in the ovalbumin-induced allergic airway in animal model. It also enhances production of IL-10 and suppresses IL-4 and IL-13 cytokines (Saw *et al*., 2012). Nafamostat is a potent serine protease inhibitor that inhibits inflammatory response in the dextran sulphate sodium-induced colitis model by suppressing mast cell chymases and impeding inflammatory infiltration at the site of inflammation (Cho *et al*., 2011).

Neutrophil elastase has also been shown to regulate the release of CXCL8, induced by signalling through Toll-like receptor 4 (TLR4), which is the main receptor for lipopolysaccharide (LPS) (Walsh D. E. *et al*., 2001 & Devaney J. M. *et al.,* 2003). Neutrophil elastase-induced upregulation of CXCL8 expression in human bronchial epithelial cells depends on the proteolytic activity of neutrophil elastase. The application of serine-protease inhibitor phenylmethylsulphonyl fluoride (PMSF) abolished this specific activation of CXCL8. Similarly, pre-treatment of macrophage inflammatory protein 2 (MIP-2) with an inhibitor of neutrophil elastase decreased LPS induced CXCL2 production by the human macrophage cell line RAW 264.7 in a dose-dependent manner (Pham, 2006).

All these studies provide evidence about the therapeutic potential of serine protease inhibitor in inhibiting or regulating the activity of serine proteases secreted by neutrophils. In this study protease inhibition from plant source were isolated, purified and characterized for their biological activity, specifically as an anti-inflammatory agent.

Objectives of the present investigation are;

1. Isolation and purification of serine protease inhibitor from selected plant seeds.
2. Effect of purified inhibitor on various proteases expressed during inflammation.
3. Molecular studies to understand the potential of protease inhibitor.

The following studies were performed towards achieving these objectives;

(i) Isolation, purification and characterization of serine protease inhibitor from seeds sources *viz*., *Dolichos biflorus (Db), Caesalpinia decapetala (Cd) and Senna siamea (Cs)* belongs to fabaceae family.

(ii) *In-vitro* inhibitory studies of isolated serine protease inhibitors.

(iii) Structural and molecular interaction studies employing spectroscopic and docking studies.

The thesis is organized into four chapters:

Chapter 1. Introduction

The first chapter deals with the general introduction and scope of the present investigation. Prevalence of serine proteases in Leguminosae family, their biological role and the structural studies of few serine proteases are introduced. Literature related to the role of serine proteases involved in physiological processes, immunological responses, and pro-inflammatory actions are reviewed. Serine proteases, the major contents of neutrophils, which are released at sites of inflammation and their role in treatment of inflammatory diseases by modifying various inflammatory pathways is described. This chapter ends with the aim and scope of investigation.

Chapter 2. Materials and Methods

Chapter 2 presents the details on the materials, methodology, and chemicals used for the current investigation and experimental procedure of the present study. This chapter briefly explains the collection of plant source, isolation, and purification of serine protease inhibitors from selected plant source, characterization of the inhibitor, *in-vitro* trypsin, chymotrypsin, and elastase inhibitory activity assessment by spectroscopic method (Kakade *et al.,* 1969) is explained.

Effect of purified inhibitor on proteases expressed during inflammation is studied using inflammatory cell line model. Lung epithelial cell line (A549) and murine macrophage cell line (RAW264.7), is chosen and the effect of purified inhibitor on cell viability and its effect on expression of various protease in the presence of inhibitors is studied.

Statistical analysis is carried out wherever it was needed. All values are expressed as Mean ± SD. Comparison between the control and sample are performed by analysis of variance (ANOVA) using Graph Pad Prism v5.0 software.

Chapter 3. Results and Discussion

Third chapter explains the results and discussion to support the objectives of this study. This chapter is organized into three sections as follows.

3.1. Isolation purification of serine protease inhibitors from plant source

The serine protease inhibitor (SPI) from the seeds of *Db, Cd* and *Cs* are purified employing conventional chromatography. The crude extract is initially subjected to ammonium sulphate precipitation to precipitate the proteins. The precipitated sample subjected to dialysis and applied on anion exchange and gel filtration chromatography, sequentially or by affinity chromatography. To achieve purification, molecular mass of purified SPI's is determined on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and by MALDI-TOF/MS analysis.

Inhibition of caseinolytic activity of SPI's are assessed by casein zymography (Felicioli et al., 1997). Characterization and kinetics *in-vitro* studies of purified SPI's are done using trypsin and elastase enzyme (Kakade *et al*., 1969).

2. Effect of purified inhibitor on various proteases expressed during inflammation

The effect of purified SPI's on various proteases (tryptase, elastase, and trypsin) expressed during inflammation was assessed by using *in-vitro* inflammatory cell line model. The Lung epithelial cell line (A549) and murine macrophage (RAW264.7) cell line were used to check the various protease level and their inhibition by using SPI. Cell viability was measured by 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) according into the method of (Sim *et al.,* 2016). Effect of purified inhibitor on tryptase, elastase, tyrosinase, and trypsin activity were assessed according into the method of (Lavens, .et.al, 1993) with some modification. Our findings clearly reveal that *CdTI*, *CsTI* and *DbTI* have significant acts as an anti-proliferatory on adenocarcinoma cells as well as suppress the pro-inflammatory markers, which is further addressed. These findings have led to PIs being classified as therapeutic potential due to their capacity to prevent and cure antiproliferative activity of cancer cell lines. The purified inhibitor shows to be a promising candidate as anti-inflammatory agent by targeting the serine proteases.

3. Molecular studies to understand the potential of protease inhibitor

The structural and molecular interaction of purified SPI's was analyzed in this section. N-terminal amino acid sequence of PI's was analysed for sequence homolog. *CdTI* had the homology with Kunitz type inhibitor where as *DbTI* had the homology with Bowman-Birk inhibitors derived from legume family as analysed with the Genbank database. 3 D structure of PI's was modelled by using MODELLER and ProSAII (Protein Structure Analysis II) programmer is used to structure prediction, refinement,

and validation of experimental protein structures. The secondary structure of purified PI's was analysed by using the Ramachandran plot (*CdTI* and *DbTI*) revealing 2 disulphide bonds and 1 helix for *CdTI* and 7 disulphide bonds were seen in *DbTI* modelled structure. The modelled structure was investigated for protease-protease inhibitor interactions using human trypsin (PDB ID: 1TRN) and human elastase (PDB ID: 1HNE). The interacted model shows similar results as shown in in-vitro studies. Based on this model, we conclude that purified PI's have potent anti-inflammatory activity against serine proteases expressed during inflammatory conditions.

The above topics are explained in details in the thesis.

Chapter 4. Summary and conclusion

- Legume seeds being rich in proteins express serine protease inhibitors for their defense. The serine protease inhibitors were purified and characterized for their physical, chemical molecular and biological properties
- From the selected legume plants, serine protease inhibitor was isolated and purified.
- Protein content, molecular mass, substrate gel assay, and kinetics were done to characterize for purified SPI's.
- *In-vitro* inhibitory studies were done for SPI's against trypsin, chymotrypsin, elastase and *in-vitro* cell line studies to assess the inhibitory effect of SPI's on proteases over expressed during inflammation were done using A549 and RAW2647 cell lines.
- Structural and molecular studies of SPI were done by molecular docking techniques.
- The secondary structure of purified PI's was analysed by using the Ramachandran plot (*CdTI* and *DbTI*) revealing 2 disulphide bonds and 1 helix for *CdTI* and 7 disulphide bonds were seen in *DbTI* modelled structure

- The modelled structure was investigated for protease-protease inhibitor interactions using human trypsin (PDB ID: 1TRN) and human elastase (PDB ID: 1HNE).
- The interacted model shows similar results as shown in *in-vitro* studies.
- Based on this model, we conclude that purified PI's have potent anti-inflammatory activity against serine proteases expressed during inflammatory conditions.
- The studies shows that protease inhibitors purified from this study could be potential molecules as anti-inflammatory by targeting the over expressed proteases.

List of Publication

1. Sachin Honnenahalli Rajegowda and Sneha Rani Athahalli Honnagirigowda. (2022) 'In-vitro anti-inflammatory activity of serine protease inhibitor from Cassia siamea and Dolichos biflorus: A comparative study', ***Cell Biochemistry and Function***. John Wiley & Sons, Ltd. DOI: 10.1002/CBF.3758.

2. Sachin H R, M.N. Sharath Kumar, S. Devaraja, and A.H. Sneharani**.** Anticoagulant and antiplatelet activities of novel serine protease purified from seeds of *Cucumis maderaspatensis*. ***3Biotech*-2021:11(1).** DOI: 10.1007/s13205-020-02565-y.

3. Sachin H. R., Prabhudev S. H., Ganesh Prasad D. N., and A. H. Sneharani. Immobilization of trypsin enzyme on silver nanoparticles. ***Biomedicine*- 2020: 40(2):188-191.**

4. Sachin H R, Hamed A Ghramh, Khalid Ali Khan and Sneha Rani A H. Anti-proinflammatory effect of a novel Kunitz type inhibitor purified from Caesalpinia decapetala seeds **(Communicated).**

5. Sachin H R, Jagadeesha Poyya, Ganeshprasad D N and Sneha Rani A H. Molecular docking studies of Bowman-Birk Inhibitor and Kunitz-Inhibitor towards serine protease **(Communicated).**

Poster presentation

1. Sachin H.R, Prabhudev S.H, and Sneha Rani A.H. "**Comparative *In-Vitro* Anti-Inflammatory Activity of Serine Protease Inhibitor from Cassia siamea and Dolichos biflorus''**, Participated in the International Conference on Contemporary Researches in Engineering, Science, Management & Arts (ICCRESMA) - 27-29 January 2022 Powered By CRT, NFED, Coimbatore, Tamil Nadu, India.

2. Sachin H.R and Sneha Rani A.H. "**'Structural and Molecular Studies of Serine Protease inhibitor from *Caesalpinia decapetela* seeds' in the "Research Scholars Meet – 2021" (RSM-2021) on 23RD & 24TH September, 2021 organized by the School of Biotechnology and Bioinformatics, D. Y. Patil Deemed to be University, Navi Mumbai.**

3. Sachin H.R, Prabhudev S.H, and Sneha Rani A.H. **Silver nanoparticles as a matrix for enzyme immobilization**, Participated in International conference on "current concepts on the role of Indian medicine and phytoceuticals in maintenance of health" organized by Dept. of Biochemistry, Jnana Kaveri, P G Centre Chikka Aluvara Mangalore University on Nov-2018.

Symposia Attended

1. Participated In the International conference on "Current concepts on the role of Indian Medicine and Phytoceuticals in Maintenance of Health" organized by Dept. of Biochemistry, Mangalore University from 15^{th}-17^{th} Nov.2018.
2. Participated in GIAN (Global Initiative for Academic Network) Organized by Dept. of Biochemistry, Mangalore University from 6^{th} to 11^{th} November 2017.
3. Participated in 'Trends in Bioactive natural products and health care (BNPHC) organized by Dept. of Biochemistry, PG Centre Chikka Aluvara, Mangalore University on 6^{th}& 7^{th} Sep-2017.

4. Participated in Symposium on science journalism and science communication organized by CSIR-CFTRI in association with Vignan prasar, New Delhi on Aug 13, 2019 at CSIR-CFTRI, Mysore, India.

Work Shops

1. Participated in a workshop on **"Basic of cell culture and application in drug discovery**" organized by Dept. of Biochemistry, Jnana Kaveri PG Centre Chikka Aluvara Mangalore University on Nov-2018.

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